# STARCH GRANULES CONTAINING A RECOMBINANT POLYPEPTIDE OF INTEREST, A METHOD OF OBTAINING THEM, AND THEIR USES

The present invention relates to starch granules containing a recombinant polypeptide of interest, a method of obtaining them, as well as their uses, especially in pharmaceutical compositions.

Starch is one of the world's most important sources of polysaccharides, occurring in particular in plants (maize, potato, wheat, rice, barley, etc.), algae, micro-algae etc. Starch occurs in the form of granules that are insoluble in water, the size of which can vary from 0.1 to several tens of μm in diameter depending on its origin (plants, algae or micro-algae) or even the genotype of the plant in question. Thus, the sizes of these granules vary from 0.1 μm in diameter to more than 50 μm in diameter. Furthermore, the degrees of crystallinity of these granules range from 0% (for granules rich in amylose) to over 30%. There are three or four crystalline types (A, B, C, V). The granule grows by the laying-down of alternately amorphous and semicrystalline layers starting from the centre of the starch granule.

Starch contains several distinct polysaccharide fractions, composed of glucans bound at α-1,4 and branched at α-1,6. More particularly, starch consists of two glucose polymers: amylose on the one hand, the minor fraction of the granule (about 20-30 wt.%), of low molecular weight, with little branching (< 1% of α-1,6 bonds) and amylopectin on the other hand, the major fraction of the granule (70-80 wt.%), of high molecular weight and highly branched (5% of α-1,6 bonds). Amylose is not necessary for the development of crystallinity of the starch granule; it is now known that it is amylopectin that is responsible for the crystallinity of the starch granule.

In biological terms, strictly speaking starch only occurs in the plant kingdom, and more specifically in the chloroplasts or in the non-photosynthetic granules of the eukaryotic plant cell. Two types of starch can be synthesized by plants: temporary or photosynthetic starch (synthesis of which takes place at the level of the chloroplasts), and reserve starch (synthesis of which takes place at the level of the amyloplasts).

Synthesis of starch in plants involves a whole panoply of enzymes taking part in biosynthesis of the precursor ADP-glucose, scaffolding of the amylose and amylopectin molecules and, finally, degradation of the starch granule.

The first stage in the biosynthesis of starch is the production of the precursor ADP-glucose with the involvement of the two enzymes: phosphoglucomutase (PGM) and ADP-glucose pyrophosphorylase (AGPase).

The second stage in the biosynthesis of the starch granule also involves two types of enzymes, mainly taking part in the synthesis of amylose and amylopectin: starch synthases (or adenosine diphosphate glucose α-1,4-glucan α-4-glucosyltransferases) and branching enzymes (or α-1,4-glucan-6-glucosyltransferases). The starch synthases catalyse the transfer of the glucose residue from ADP-glucose onto growing chains of glucans by creating an O-glycosidic bond of type α-1,4. Then the branching enzymes hydrolyse an α-1,4 bond of an elongating glucan, and then join the fragment thus released onto the remainder of the glucan by means of an α-1,6 bond.

With regard to starch degradation, there are two main families of degrading enzymes: the hydrolytic enzymes (hydrolases) on the one hand, such as α-amylases (endomylases), β-amylases (exomylases), γ-amylases (amyloglucosidases), D-enzymes (glucosyltransferases), R-enzymes (debranching enzymes), α-glucosidases (maltases) and, on the other hand, the phosphorolytic enzymes (or starch phosphorylases).

Several isoforms of starch synthases occur together in higher plants. The main difference between these isoforms relates to their solubility (i.e. they are dissolved in the plastid stroma in plants) or to the fact that they are bound to the starch granule.

The starch synthases bound to the starch granule (or GBSS: Granule Bound Starch Synthases) occur in close association with starch. Several isoforms of GBSS have been isolated in maize, pea, potato or wheat (*MacDonald and Preiss, 1985; Smith, 1990; Dry et al., 1992; Denyer et al., 1995*). In all cases, GBSSI is the main isoform; the part played by this isoform in the biogenesis of the starch granule is the formation of amylose (*Tsai, 1974; Hovenkamp-Hermelink et al., 1987; Delrue et al., 1992; Denyer et al., 1995*). A mutation at the loci *WX* of cereals, *AMF* of the potato, and *LAM* of the pea, combines disappearance of GBSSI with complete collapse of the amylose fraction of starch. A cDNA corresponding to the "Waxy protein" (through a misuse of language, the term "Waxy protein" is employed to designate the GBSSI in plants, thus distinguishing it from other GBSS) has been isolated in wheat, barley, maize, rice, potato and pea. Comparisons of the relative protein sequences show there is considerable homology between the different species (*Ainsworth et al.*, 1993).

GBSSI is not the only starch synthase bound to the starch granule. Other isoforms are found bound to the starch granule in pea, potato, maize or wheat (*Smith, 1990; Dry et al., 1992; Mu et al., 1994; Denyer et al., 1995*). However, the roles of these various isoforms are not yet clear. Furthermore, most of them are also found in the soluble phase.

The soluble starch synthases (SS) are not bound to the starch granule, but are found in soluble form in the plastid stroma of plants. As with the bound forms, several forms of soluble starch synthases occur in the higher plants. For example, three isoforms of soluble starch synthases (SSI, SSII and SSIII) have been detected in the potato tuber.

cDNA's corresponding to various forms of soluble starch synthases have been cloned in higher plants (*Baba et al., 1993; Dry et al., 1992; Edwards et al., 1995; Abel et al., 1996; Marshall et al., 1996; Gao et al., 1998*). Sequence comparison flowing from this clearly shows the presence of three regions that are highly conserved across the isoforms, whether within a single species or between species of higher plants.

Recent research by the Inventors made it possible to establish that soluble starch synthase II (SSII) from *Chlamydomonas reinhardtii* is mainly involved in the formation of crystals of the amylopectin molecule.

On the other hand, GBSSI does not take part in the construction of amylopectin crystals. GBSSI activity has never been detected in the soluble phase. GBSSI is intimately associated with the starch granule. However, in contrast to amylase, no unit binding to starch has been found in the GBSSI sequences described so far. Accordingly, the mechanism controlling the binding of GBSSI to the starch granule is unknown.

Starch synthases are of particular interest in that these enzymes might make it possible to transport a recombinant peptide of interest towards the plastids where the biosynthesis of starch granules takes place. Thus, the transformation of plants with sequences coding for fusion peptides between a starch synthase and a peptide of interest would make it possible to obtain starch granules in large quantity, from which the said peptide of interest could be recovered.

It was with this objective that the authors of International Application WO 98/14601 (Exseed Genetics) described nucleotide sequences coding for fusion proteins in which the polypeptide of interest is bound to the amino terminal end of a starch synthase selected from the group comprising soluble starch synthases I, II and III (SSI, SSII, SSIII), granule bound starch synthases (GBSS), branching enzymes I, IIa and IIBb

and the glucoamylases. However, no method of transformation of plants by means of the sequences described in that application, and hence of obtaining starch granules transformed by the said sequences, is illustrated in detail.

The present invention arises from the demonstration by the inventors that only the transformation of plants with nucleotide sequences coding for fusion polypeptides in which the polypeptide of interest is bound to the carboxy terminal end of the starch synthase makes it possible to obtain starch granules containing the said peptide of interest.

One of the aims of the present invention is to provide novel nucleotide sequences coding for fusion proteins capable of transporting a peptide of interest towards the site of biosynthesis of the starch granules in plant cells (including the cells of algae or micro-algae).

Another aim of the present invention is to provide plants that have been transformed by means of the aforementioned nucleotide sequences, the said plants producing starch containing a polypeptide of interest.

Another aim of the present invention is to provide starch granules containing a polypeptide of interest.

Another aim of the present invention is to provide a method of preparation of these starch granules.

Another aim of the present invention is to provide a method of preparation of a recombinant polypeptide of interest starting from these starch granules.

Another aim of the present invention is to provide compositions, especially pharmaceutical or for foodstuffs, containing the aforementioned starch granules.

Another aim of the present invention is to provide a method of biotransformation of starch granules when the said peptide of interest that is used is capable of transforming starch.

The invention will be illustrated below with the aid of the following diagrams:

– Fig. 1: cDNA coding for the carboxy terminal part of the GBSSI of *Chlamydomonas reinhardtii*,

– Fig. 2: cDNA coding for the GBSSI of *Chlamydomonas reinhardtii*, and peptide sequence of the GBSSI of *Chlamydomonas reinhardtii*.

The present invention relates to any recombinant nucleotide sequence characterized in that it comprises, in the direction 5'→3', a nucleotide sequence coding for an adenosine diphosphate glucose α-1,4-glucan α-4-glucosyltransferase or starch

synthase (EC 2.4.1.21), or for a protein derived from this enzyme, especially by suppression, addition or substitution of one or more amino acids, the said starch synthase or derived protein having the property of migrating to the sites of biosynthesis of starch granules in plant cells and of attaching to the starch granules, the said nucleotide sequence coding for the enzyme or aforementioned protein being positioned upstream of a nucleotide sequence coding for a peptide or polypeptide of interest.

By starch synthase we mean, in the foregoing and hereinafter, any protein having the property of migrating to the sites of biosynthesis of starch granules in plant cells and of attaching to the starch granules, whether or not this starch synthase has conserved its enzymatic activity within the fusion polypeptide, encoded by an aforementioned recombinant nucleotide sequence, between the said starch synthase and the said polypeptide of interest.

Preferably, the nucleotide sequence coding for a starch synthase, or for a derived protein as defined above, is selected from those coding for a starch synthase bound to the starch granule GBSS that occurs in particular in plants, algae or micro-algae, and even more advantageously for an isoform GBSSI, or for a protein derived from this GBSS, or GBSSI, as defined above.

The invention relates more particularly to any recombinant nucleotide sequence as defined above, characterized in that the nucleotide sequence coding for a starch synthase, and more particularly for a GBSS, and especially for a GBSSI, is such as is obtained by screening a cDNA library prepared from cells that are likely to contain this enzyme, especially from cells of plants, algae or micro-algae, by means of an antiserum containing antibodies specifically recognizing the said starch synthase coded by one or more cDNA in the library, when the said starch synthase is expressed by a suitable cloning vector, the said antiserum being obtained by immunization of an animal, such as a rabbit, with starch extracted from the aforementioned cells.

The invention relates more particularly to any recombinant nucleotide sequence as defined above, characterized in that the nucleotide sequence coding for a starch synthase, or for a derived protein, is selected from:

– the nucleotide sequence of the cDNA of about 2900 to 3100 base pairs, and of which the 1696 base pairs of the 3' end are shown in Fig. 1, the said nucleotide sequence:

. coding for the GBSSI of *Chlamydomonas reinhardtii* of about 640 to 680 amino acids, especially of about 660 amino acids, of which the amino terminal

end corresponds to the following succession of amino acids: ALDIVMVAAEVAPGGKTGGLGDV, or ALDIVMVAAEVAPWSKTGGLGD-V, and of which the carboxy terminal end corresponds to the succession of amino acids shown in Fig. 1,

. and being obtained by screening a cDNA library prepared from cells of *Chlamydomonas reinhardtii*, by means of an antiserum obtained by immunization of rabbits with the starch extracted from the aforementioned cells of *Chlamydomonas reinhardtii*,

– or a nucleotide fragment of the aforementioned cDNA, coding for a peptide fragment of the GBSSI of *Chlamydomonas reinhardtii*, the said peptide fragment comprising the whole of the amino terminal part of the said GBSSI, and being delimited at its carboxy terminal end by the amino acid located in one of the positions 25 to 238, or in one of the positions 118 to 238, of the amino acid sequence shown in Fig. 1,

– or a nucleotide sequence derived by degeneration of the genetic code of the nucleotide sequence of the aforementioned cDNA, or of an aforementioned nucleotide fragment of the latter, and coding for the aforementioned GBSSI of *Chlamydomonas reinhardtii*, or for an aforementioned peptide fragment of the latter,

– or a nucleotide sequence derived from an aforementioned nucleotide sequence or fragment, especially by substitution, suppression or addition of one or more nucleotides, and encoding a peptide sequence derived from the aforementioned GBSSI of *Chlamydomonas reinhardtii*, or derived from an aforementioned peptide fragment of the latter, and having the property of attaching to the starch granules, the said derived nucleotide sequence preferably having a homology of at least about 50%, and preferably of at least about 70%, with the aforementioned nucleotide sequence or fragment,

– or a nucleotide sequence capable of hybridization with one of the aforementioned nucleotide sequences or fragments, especially in the strict conditions of hybridization defined later,

the property possessed by a starch synthase, or a fragment or a protein derived from the latter as defined above, of being able to attach to the starch granules, being measurable by the following technique: extraction of the proteins from the starch granules, for example according to the method described in detail below, and detection of the presence of the said starch synthase, or of a fragment or of a protein derived from the latter as defined above, especially by polyacrylamide gel electrophoresis according to the technique described in detail later.

The invention relates more particularly to any recombinant nucleotide sequence as defined above, characterized in that the aforementioned nucleotide sequence coding for a starch synthase, or for a derived protein, is more particularly selected from:

– the nucleotide sequence of cDNA shown in Fig. 2, corresponding to SEQ ID NO : 1 in the sequence list given later, the said nucleotide sequence coding for the GBSSI of *Chlamydomonas reinhardtii*,

– any fragment as defined above of the nucleotide sequence SEQ ID NO : 1 shown in Fig. 2, and more particularly any sequence of which the nucleotide of the 5' end corresponds to that located in one of the positions 1 to 186 of SEQ ID NO : 1, and of which the nucleotide of the 3' end corresponds to that located in one of the positions 1499 to 3117 of SEQ ID NO : 1, especially:

. the sequence SEQ ID NO : 2 delimited by the nucleotides located in positions 15 to 2138 of SEQ ID NO : 1, coding for the GBSSI of *Chlamydomonas reinhardtii* in the form of pre-protein of 708 amino acids (SEQ ID NO : 3) delimited by the amino acids located at positions 1 and 708 of the peptide sequence shown in Fig. 2,

. the sequence SEQ ID NO : 4 delimited by the nucleotides located at positions 186 to 2138 of SEQ ID NO : 1, coding for the GBSSI of *Chlamydomonas reinhardtii* in the form of a mature protein of 651 amino acids (SEQ ID NO : 5) delimited by the amino acids located at positions 58 and 708 of the peptide sequence shown in Fig. 2,

. the sequence SEQ ID NO : 6 delimited by the nucleotides located at positions 186 to 1499 of SEQ ID NO : 1, coding for a fragment of 438 amino acids (SEQ ID NO : 7) delimited by the amino acids located at positions 58 and 495 of the peptide sequence of the GBSSI of *Chlamydomonas reinhardtii* shown in Fig. 2,

. the sequence SEQ ID NO : 8 delimited by the nucleotides located at positions 186 to 1778 of SEQ ID NO : 1, coding for a fragment of 531 amino acids (SEQ ID NO : 9) delimited by the amino acids located at positions 58 and 588 of the peptide sequence of the GBSSI of *Chlamydomonas reinhardtii* shown in Fig. 2,

– or a nucleotide sequence derived by degeneration of the genetic code of the aforementioned nucleotide sequences, and coding for the aforementioned GBSSI of *Chlamydomonas reinhardtii*, or for an aforementioned peptide fragment of the latter,

– or a nucleotide sequence derived from an aforementioned nucleotide sequence or fragment, especially by substitution, suppression or addition of one or more nucleotides, and encoding a peptide sequence derived from the aforementioned GBSSI of *Chlamydomonas reinhardtii*, or derived from an aforementioned peptide fragment of the latter, and having the property of attaching to the starch granules, the said derived nucleotide sequence preferably having a homology of at least about 50%, and preferably of at least about 70%, with the aforementioned nucleotide sequence or fragment,

– or a nucleotide sequence capable of hybridizing with one of the aforementioned nucleotide sequences or fragments, especially in the strict conditions of hybridization defined above.

The invention relates more particularly to any recombinant nucleotide sequence as defined above, characterized in that the nucleotide sequence coding for a peptide or polypeptide of interest is selected from those encoding biologically active peptides, especially peptides of therapeutic interest or that can be used in the agricultural and food industry.

The invention also relates to any recombinant nucleotide sequence as defined above, characterized in that the nucleotide sequence coding for a peptide or polypeptide of interest is selected from those encoding enzymes that are able to transform starch, such as the enzymes that interact with the α-glucans including various hydrolases, phosphorylases, α-1,4 glucanotransferases, branching enzymes, amylases, and especially the heat-resistant hydrolases obtained from extremophiles such as the archaebacteria that are active at temperatures above 40°C.

The invention also relates to any recombinant nucleotide sequence as defined above, characterized in that it comprises a nucleotide sequence coding for a cleavage site, the said nucleotide sequence being positioned between the nucleotide sequence coding for a starch synthase, or a protein derived from the latter, and the nucleotide sequence encoding the polypeptide of interest.

As an illustration, the nucleotide sequence coding for a cleavage site is selected from the sequences coding for a peptide sequence of the aspartyl-proline type, which is very unstable at acid pH, or coding for a small peptide sequence recognized specifically by a protease, such as trypsin, chymotrypsin, pepsin, collagenase, thrombin, alasubtilisin, or recognized by chemical compounds such as cyanogen bromide.

The invention also relates to any recombinant nucleotide sequence as defined above, characterized in that it comprises a promoter located upstream of the nucleotide

sequence coding for a starch synthase, or a protein derived from the latter, as well as a sequence coding for transcription termination signals located downstream of the nucleotide sequence encoding the polypeptide of interest.

Among the transcription promoters suitable for use within the scope of the present invention, we may mention:

– for prokaryotic promoters, the Lac or T7 promoters,

– for the eukaryotic promoters of higher plant type, the promoter 35S CaMV, or any type of promoter of plant origin,

– in the case of the transformation of micro-algae, the promoter used can be that of the *ARG7* gene encoding arginosuccinate lyase or the promoter of the *NIT1* gene encoding nitrate reductase.

The invention also relates to any recombinant vector, especially of the plasmid, cosmid or phage type, characterized in that it contains a recombinant nucleotide sequence according to the invention as defined above, inserted in a site that is non-essential for its replication.

The invention also relates to any cellular host, transformed by a recombinant vector as defined above, especially any bacterium such as *Agrobacterium tumefaciens*, and comprising at least one recombinant nucleotide sequence according to the invention.

The invention also relates to any fusion polypeptide characterized in that it comprises:

– in the N-terminal position, a starch synthase, or a protein derived from that enzyme, especially by suppression, addition or substitution of one or more amino acids, the said starch synthase or derived protein having the property of migrating to the sites of biosynthesis of the starch granules in plant cells and of attaching to the starch granules,

– and, in the C-terminal position, a peptide or polypeptide of interest,

the C-terminal part of the amino acid sequence of the starch synthase, or of the derived protein, being thus bound to the N-terminal part of the peptide sequence of interest, the said fusion polypeptide being encoded by a recombinant nucleotide sequence as defined above according to the invention.

The invention relates more particularly to any fusion polypeptide as defined above, characterized in that it includes, in the N-terminal position, a GBSS that occurs

in particular in plants, algae or micro-algae, and more particularly an isoform GBSSI, or a protein derived from the latter as defined above.

The invention relates more particularly to any fusion polypeptide as defined above, characterized in that the starch synthase is selected from:

– the GBSSI of *Chlamydomonas reinhardtii* of about 640 to 680 amino acids, of which the amino terminal end corresponds to the following succession of amino acids: ALDIVMVAAEVAPGGKTGGLGDV, or ALDIVMVAAEVAPWSKTGGLGDV, and the carboxy terminal end corresponds to the succession of amino acids shown in Fig. 1, the said GBSSI being encoded by the nucleotide sequence obtained by screening a cDNA library prepared from cells of *Chlamydomonas reinhardtii*, by means of an antiserum obtained by immunization of rabbits with the starch extracted from the aforementioned cells of *Chlamydomonas reinhardtii*,

– or a peptide fragment of the GBSSI of *Chlamydomonas reinhardtii*, the said peptide fragment comprising the whole of the amino terminal part of the said GBSSI, and being delimited at its carboxy terminal end by the amino acid located in one of the positions 25 to 238, or in one of the positions 118 to 238, of the amino acid sequence shown in Fig. 1,

– or a peptide sequence derived from an aforementioned peptide sequence or fragment, especially by substitution, suppression or addition of one or more amino acids, and having the property of attaching to the starch granules, the said derived peptide sequence preferably having a homology of at least about 60%, and advantageously of at least about 80%, with the aforementioned peptide sequence or fragment,

the property possessed by the GBSSI of *Chlamydomonas reinhardtii*, or a fragment or a protein derived from the latter as defined above, of being able to attach to the starch granules, being measurable by the technique described above.

The invention relates more particularly to any fusion polypeptide as defined above, characterized in that the starch synthase defined above is selected more particularly from:

– the peptide sequence SEQ ID NO : 3 delimited by the amino acids located at positions 1 to 708 in Fig. 2, corresponding to the GBSSI of *Chlamydomonas reinhardtii* in the form of a pre-protein of 708 amino acids,

– any fragment as defined above from the peptide sequence SEQ ID NO : 3 shown in Fig. 2, and more particularly any sequence of which the amino acid of the

amino terminal end corresponds to that located in one of the positions 1 to 58 of SEQ ID NO : 3, and of which the amino acid of the carboxy terminal end corresponds to that located in one of the positions 495 to 708 of SEQ ID NO : 3, especially:

. the sequence SEQ ID NO : 5 delimited by the amino acids located at positions 58 to 708 of SEQ ID NO : 3, corresponding to the GBSSI of *Chlamydomonas reinhardtii* in the form of a mature protein of 651 amino acids,

. the sequence SEQ ID NO : 7 delimited by the amino acids located at positions 58 to 495 of SEQ ID NO : 3, corresponding to a fragment of 438 amino acids of the peptide sequence of the GBSSI of *Chlamydomonas reinhardtii* shown in Fig. 2,

. the sequence SEQ ID NO : 9 delimited by the amino acids located at positions 58 to 588 of SEQ ID NO : 3, corresponding to a fragment of 531 amino acids of the peptide sequence of the GBSSI of *Chlamydomonas reinhardtii* shown in Fig. 2,

– or a peptide sequence derived from an aforementioned peptide sequence or fragment, especially by substitution, suppression or addition of one or more amino acids, and having the property of attaching to the starch granules, the said derived peptide sequence preferably having a homology of at least about 60%, and advantageously of at least about 80%, with the aforementioned peptide sequence or fragment,

the property possessed by the GBSSI of *Chlamydomonas reinhardtii*, or a fragment or a protein derived from the latter as defined above, of being able to attach to the starch granules, being measurable by the technique described above.

The invention relates more particularly to any fusion polypeptide as defined above, characterized in that the polypeptide of interest is selected from the biologically active peptides, especially the peptides of therapeutic interest or that can be used in the agricultural and food industry.

The invention also relates to any fusion polypeptide as defined above, characterized in that the polypeptide of interest is selected from the enzymes that are able to transform starch, such as the enzymes that interact with the α-glucans, including various hydrolases, phosphorylases, α-1,4-glucanotransferases, branching enzymes, amylases, and especially the heat-resistant hydrolases obtained from extremophiles such as the archaebacteria that are active at temperatures above 40°C.

The invention also relates to any fusion polypeptide as defined above, characterized in that it contains a cleavage site, as described above, positioned between on the one hand the starch synthase or a protein derived from the latter, and on the other hand the polypeptide of interest.

The invention also relates to genetically transformed plant cells, containing one or more recombinant nucleotide sequences as described above, integrated in their genome or maintained in a stable manner in their cytoplasm, the said plant cells being selected from the cells of plants, algae or micro-algae, capable of producing starch.

The invention also relates to transgenic plant cells as described above containing one or more fusion polypeptides defined above within the starch granules contained in the said plant cells.

The invention relates more particularly to the aforementioned transgenic plant cells, transformed with a recombinant nucleotide sequence containing the nucleotide sequence of cDNA of about 2900 to 3100 base pairs, and of which the 1696 base pairs of the 3' end are shown in Fig. 1, the said nucleotide sequence coding for the GBSSI of *Chlamydomonas reinhardtii* described above, or containing a fragment or a derived sequence as described above from the aforementioned cDNA.

The invention relates even more particularly to the aforementioned transgenic plant cells, transformed with:

– the cDNA nucleotide sequence shown in Fig. 2, the said nucleotide sequence coding for the GBSSI of *Chlamydomonas reinhardtii*,

– any fragment as defined above of the nucleotide sequence shown in Fig. 2,

– or a derived nucleotide sequence, as defined above, from the aforementioned nucleotide sequences,

– or a nucleotide sequence capable of hybridization with one of the aforementioned nucleotide sequences or fragments, especially in the strict conditions of hybridization defined above.

The invention also relates to genetically transformed plants, algae or micro-algae, or parts, especially flowers, fruits, leaves, stems, roots, seeds, or fragments of these plants, algae or micro-algae, containing at least one recombinant nucleotide sequence as defined above integrated in the genome or maintained in a stable manner in the cytoplasm of the cells of which they are composed.

The invention also relates to genetically transformed plants, algae or micro-algae, or parts, or fragments of these plants, algae or micro-algae, as defined above, containing

one or more fusion polypeptides as described above within the starch granules contained in the plant cells of which they are composed.

Among the plants, algae or micro-algae transformed within the scope of the present invention, we may mainly mention wheat, maize, potato, rice, barley, amaranth, algae of the genus *Chlamydomonas*, especially *Chlamydomonas reinhardtii*, algae of the genus *Chlorella*, especially *Chlorella vulgaris*, or single-celled algae of the genus *Dunaliella* (as described in the work "*Dunaliella*: Physiology, Biochemistry, and Biotechnology (1992), Mordhay Avron and Ami Ben-Amotz Publishers, CRC Press Inc., Boca Raton, Florida, USA").

The invention relates more particularly to the aforementioned transgenic plants, algae or micro-algae, transformed with a recombinant nucleotide sequence containing the cDNA nucleotide sequence of about 2900 to 3100 base pairs and of which the 1696 base pairs of the 3' end are shown in Fig. 1, the said nucleotide sequence coding for the GBSSI of *Chlamydomonas reinhardtii* described above, or containing a fragment or a derived sequence such as are described above of the aforementioned cDNA.

The invention relates even more particularly to the aforementioned transgenic plants, algae or micro-algae, transformed with:

– the cDNA nucleotide sequence shown in Fig. 2, the said nucleotide sequence coding for the GBSSI of *Chlamydomonas reinhardtii*,

– any fragment as defined above of the nucleotide sequence shown in Fig. 2,

– or a derived nucleotide sequence, as defined above, of the aforementioned nucleotide sequences,

– or a nucleotide sequence capable of hybridizing with one of the aforementioned nucleotide sequences or fragments, especially in the strict conditions of hybridization defined above.

The invention also relates to starch granules characterized in that they include one or more fusion polypeptides defined above, the said starch granules being further designated by the expression "transformed starch granules" or "glucosomes".

The invention relates more particularly to the aforementioned starch granules comprising a fusion polypeptide defined above, the said fusion polypeptide containing the GBSSI of *Chlamydomonas reinhardtii* of about 640 to 680 amino acids described above, the amino terminal end of which corresponds to the following succession of amino acids: ALDIVMVAAEVAPGGKTGGLGDV, or ALDIVMVAAEVAPWSKTG-GLGDV, and the carboxy terminal end corresponds to the succession of amino acids

shown in Fig. 1, or a fragment or a derived polypeptide such as are described above of the GBSSI of *Chlamydomonas reinhardtii*.

The invention relates more particularly to the aforementioned starch granules comprising a fusion polypeptide defined above, the said fusion polypeptide containing the sequence delimited by the amino acids located at positions 1 to 708 in Fig. 2, coding for the GBSSI of *Chlamydomonas reinhardtii* in the form of a pre-protein of 708 amino acids, or any fragment as defined above of the peptide sequence shown in Fig. 2, especially any sequence in which the amino acid of the amino terminal end corresponds to that located in one of the positions 1 to 58 in Fig. 2, and in which the amino acid of the carboxy terminal end corresponds to that located in one of the positions 495 to 708 in Fig. 2, such as the fragments mentioned above.

Advantageously, the aforementioned starch granules are characterized in that they have a diameter between about 0.1 μm and several tens of μm, and in that the proportion by weight of the fusion polypeptides in these granules is between about 0.1% and 1%.

The invention also relates to any pharmaceutical composition characterized in that it includes transformed starch granules as defined above, if necessary in combination with a physiologically acceptable vehicle, the said granules containing one or more fusion polypeptides as defined above, the peptide of interest in the said fusion polypeptides possessing a defined therapeutic effect.

Advantageously the aforementioned pharmaceutical compositions of the invention are in a form that can be administered parenterally, especially intravenously, or in a form that can be administered orally.

Preferably, the aforementioned pharmaceutical compositions that can be administered parenterally are characterized in that the diameter of the starch granules is between about 0.1 μm and several μm, especially between about 0.1 μm and 10 μm, and in that the proportion by weight of the fusion polypeptides in these granules is between about 0.1% and 1%.

Starch granules as described above, with small diameters between about 0.1 μm and about 10 μm, in which the proportion by weight of fusion polypeptides is between about 0.1% and 1%, are obtained advantageously:

– from plants or cells of plants transformed within the scope of the present invention and selected for their property of producing the aforementioned starch granules naturally, the said plants being selected in particular from rice and amaranth,

– or from parts of transformed plants within the scope of the present invention, the said parts of these plants producing the aforementioned starch granules naturally, such as the leaves of the plants,

– or from plants or cells of plants transformed within the scope of the present invention, these plants being selected from plants that have mutations such that they produce starch granules of small diameters as mentioned above, especially from the mutant plants described in Buléon A. et al., 1998,

– or from plants or cells of plants transformed within the scope of the present invention, these plants being selected from the plants transformed with the aid of antisense nucleotide sequences of all or part of the gene coding for ADP-glucose pyrophosphorylase required for the synthesis of ADP-glucose in plant cells, and especially from the transformed plants described in the article by Müller-Röber B. et al., 1992.

Advantageously, in the case of pharmaceutical compositions mentioned above that can be administered parenterally, the starch granules are preferably selected from those of amorphous structure in the case when we wish to obtain rapid release of the fusion polypeptide that they contain in the patient's blood, or conversely, from those of crystalline structure when we wish to release the fusion polypeptide progressively in the blood.

By way of illustration, amorphous starch granules can be obtained from seeds transformed according to the invention at the germination stage, or from specific mutant plants such as described by Shannon J. and Garwood D., 1984, especially from the mutant cultivars such as "amylose extender" of maize or indeed all mutant cultivars of plants, algae or micro-algae whose starch is amylose-enriched.

The starch granules according to the invention of crystalline structure, advantageously have about 30 to 35% of crystals, and can be obtained from seeds of plants, especially of cereals, that have just been harvested and at maturity, or from mutant plants such as described by Shannon J. and Garwood D., 1984, especially from the mutant cultivars such as "waxy" of maize or indeed all the mutant cultivars of plants, algae or micro-algae whose starch is devoid of amylose.

The invention also relates to any pharmaceutical composition characterized in that it includes one or more fusion polypeptides as defined above, if necessary in combination with a physiologically acceptable vehicle, the peptide of interest in the said fusion polypeptides possessing a defined therapeutic effect.

The invention also relates to any food composition as described above, characterized in that it contains transformed starch granules as defined above, the said granules containing one or more fusion polypeptides as defined above, the peptide of interest in the said fusion polypeptides being usable in the food-processing field.

The invention also relates to any food composition as described above, characterized in that it contains one or more fusion polypeptides as defined above, the peptide of interest in the said fusion polypeptides being usable in the food-processing field.

The present invention also relates to any method of obtaining plant cells (from plants, algae or micro-algae), and, if necessary, from whole plants, algae or micro-algae, transformed by at least one nucleotide sequence as defined above, characterized in that it comprises:

– the transformation of plant cells, in such a way as to integrate in the genome of these cells, or maintain in a stable manner in their cytoplasm, one or more recombinant nucleotide sequences according to the invention, and cultivation of these transformed cells *in vitro*,

– if necessary, the production of transformed plants from the aforementioned transformed cells.

According to one embodiment of the aforementioned method of the invention, the transformation of plant cells can be carried out by transfer of the recombinant nucleotide sequence of the invention in the protoplasts, especially after incubation of the latter in a solution of polyethylene glycol (PEG) in the presence of divalent cations ($Ca^{2+}$) according to the method described in the article by Krens *et al.*, 1982.

Transformation of the plant cells can also be carried out by electroporation especially according to the method described in the article by Fromm *et al.*, 1986.

Transformation of the plant cells can also be carried out using a gene gun, by means of which metal particles coated with recombinant nucleotide sequences according to the invention are propelled at high velocity, thus delivering genes to the interior of the cell nucleus, especially in accordance with the technique described in the article by Sanford, 1988.

Another method of transformation of plant cells is the method of cytoplasmic or nuclear micro-injection as described in the article by De La Penna *et al.*, 1987.

According to a particularly preferred embodiment of the aforementioned method of the invention, the plant cells are transformed by putting the latter in the presence of a cellular host transformed by a vector according to the invention, as described above, the said cellular host being able to infect the said plant cells making it possible to integrate in the genome or maintain in a stable manner in the cytoplasm of the latter, recombinant nucleotide sequences of the invention initially contained in the genome of the aforementioned vector.

Advantageously, the aforementioned cellular host employed is *Agrobacterium tumefaciens*, especially according to the methods described in the articles of Bevan, 1984 and of An *et al.*, 1986, or *Agrobacterium rhizogenes*, especially according to the method described in the article by Jouanin *et al.*, 1987.

Among the plant cells capable of being transformed within the scope of the present invention, we may mention mainly the cells of wheat, maize, potato, rice, barley, amaranth, *Chlamydomonas reinhardtii*, *Chlorella vulgaris*.

According to one embodiment of the aforementioned method of the invention, the plant cells transformed according to the invention are cultivated *in vitro*, especially in bioreactors according to the method described in the article by Brodelius, 1988, in a liquid medium, or according to the method described in the article by Brodelius *et al.*, 1979, in immobilized form, or according to the method described in the article by Deno *et al.*, 1987, by culture of roots transformed *in vitro*.

According to a preferred embodiment of the aforementioned method of the invention, the transformation of plant cells is followed by a stage of obtaining transformed plants by culturing the said transformed cells in a suitable medium, and, if necessary, fertilization and recovery of the seeds of the plants obtained in the preceding stage, and cultivation of these seeds to obtain plants of the next generation.

The seeds transformed according to the invention are harvested from the aforementioned transformed plants, these plants being either those of the T0 generation, i.e. those obtained from culture of transformed cells of the invention on a suitable medium, or advantageously those of the next generations (T1, T2 etc.) obtained by self-fertilization of the plants of the preceding generation and in which the recombinant nucleotide sequences of the invention are reproduced in accordance with Mendel's laws, or the laws of extrachromosomal inheritance.

The invention also relates to a method of preparation of transformed starch granules as described above, characterized in that it comprises a stage of extraction of the starch granules from transformed plant cells or from plants, or from parts, especially flowers, fruits, leaves, stems, roots, or from fragments of these plants, transformed as mentioned above, especially by sedimentation in the conditions described later.

Preferably, the starch granules according to the invention are those obtained by extraction from transformed plants, algae or micro-algae, described above, or from parts, or fragments of these plants, algae or micro-algae, defined above, especially by sedimentation in the conditions described later.

The transformed plants used for recovering the starch granules are those of the T0 generation, or advantageously those of the next generations (T1, T2 etc.) mentioned above.

The invention also relates to a method of preparation of fusion polypeptides as defined above, characterized in that it comprises a stage of recovery, and if necessary of purification, of the fusion polypeptides from the aforementioned transformed starch granules especially in the conditions described later.

The invention also relates to a method of preparation of a peptide of interest, characterized in that it comprises the implementation of a method as described above for obtaining plant cells or transformed plants according to the invention, the said method being carried out by transformation of plant cells with the aforementioned nucleotide sequences coding for a fusion polypeptide containing a cleavage site as described above, and includes an additional stage of cleavage of the said fusion polypeptide, by means of a suitable reagent, then, if necessary, a stage of purification of the polypeptide of interest.

The invention also relates to a method of biotransformation of starch granules, characterized in that it comprises the following stages:

– transformation of plant cells as defined above with the aid of host cells described above containing one or more nucleotide sequences coding for enzymes capable of transforming starch as mentioned above,

– production of plants, algae or micro-algae transformed in such a way that their genome contains one or more nucleotide sequences described above, by culture *in vitro* of the aforementioned transformed plant cells,

– if necessary, fertilization and recovery of the seeds of the plants obtained in the preceding stage, and culture of these seeds to obtain plants of the next generation,

– extraction of starch granules from the aforementioned transformed plants, algae or micro-algae, or from parts, especially flowers, fruits, leaves, stems, roots, or from fragments of these plants, algae or micro-algae, especially by sedimentation in the conditions described later,

– if necessary, heating of the said starch granules to a temperature at which the peptide of interest of the aforementioned fusion polypeptide is capable of being active.

Preferably, when the methods described above are carried out by transformation of plant cells, the latter are transformed with the aforementioned recombinant sequences containing the cDNA nucleotide sequence of about 2900 to 3100 base pairs, and of which the 1696 base pairs of the 3' end are shown in Fig. 1, the said nucleotide sequence coding for the GBSSI of *Chlamydomonas reinhardtii* described above, and more particularly with recombinant sequences as mentioned above containing the nucleotide sequence shown in Fig. 2, or containing a fragment or a derived sequence as described above of the nucleotide sequence shown in Fig. 2. The use of these recombinant sequences containing the nucleotide sequence coding for the GBSSI of *Chlamydomonas reinhardtii* described above makes it possible advantageously to avoid the development of effects of co-suppression in the transformed plants thus obtained.

The invention also relates to a method of preparation of antibodies specifically recognizing a starch synthase bound to the starch granule, of a given plant, algae or micro-algae, by immunization of an animal, especially of a rabbit, with the starch obtained from the said plant, algae or micro-algae.

Therefore, the invention relates more particularly to a method of preparation of antibodies specifically recognizing the GBSSI, of a given plant, algae or micro-algae, by immunization of an animal, especially of a rabbit, with the starch obtained from the said plant, algae or micro-algae.

The invention also relates more particularly to a method of preparation of antibodies specifically recognizing an isoform of GBSS other than GBSSI, from a given plant, algae or micro-algae, by immunization of an animal, especially a rabbit, with the starch obtained from the said plant, algae or micro-algae having a mutation such that the expression of the GBSSI is suppressed, for example a mutation selected from the following: *sta2-29::ARG7* in *Chlamydomonas reinhardtii* (described by *Delrue et al., 1992*, mentioned above), *amf* in the potato (described by *Hovenkamp-Hermelink et al., 1987*, mentioned above), *wx* in maize, rice and wheat (described by *Tsai, 1974*, mentioned above), *lam* in the pea (described by *Denyer et al., 1995*, mentioned above).

The invention also relates to a method of obtaining starch synthase, such as GBSS, and more particularly for the isoform GBSSI, from a given plant, algae or micro-algae, by screening a cDNA library prepared from cells of the said given plant, algae or micro-algae, capable of containing this enzyme, using an antiserum containing antibodies specifically recognizing the said enzyme encoded by one or more cDNA's from the library, when the said enzyme is expressed by a suitable cloning vector, the said antiserum being obtained according to the method mentioned above.

The invention also relates to the nucleotide sequences coding for a starch synthase or for a derived protein, selected from:

– the cDNA nucleotide sequence shown in Fig. 2, corresponding to SEQ ID NO : 1 in the sequence list given later, the said nucleotide sequence coding for the GBSSI of *Chlamydomonas reinhardtii*,

– any fragment as defined above of the nucleotide sequence SEQ ID NO : 1 shown in Fig. 2, and more particularly any sequence whose nucleotide of the 5' end corresponds to that located in one of the positions 1 to 186 of SEQ ID NO : 1, and whose nucleotide of the 3' end corresponds to that located in one of the positions 1499 to 3117 of SEQ ID NO : 1, especially:

. the sequence SEQ ID NO : 2 delimited by the nucleotides located at positions 15 to 2138 of SEQ ID NO : 1, coding for the GBSSI of *Chlamydomonas reinhardtii* in the form of pre-protein of 708 amino acids (SEQ ID NO : 3) delimited by the amino acids located at positions 1 and 708 of the peptide sequence shown in Fig. 2,

. the sequence SEQ ID NO : 4 delimited by the nucleotides located at positions 186 to 2138 of SEQ ID NO : 1, coding for the GBSSI of *Chlamydomonas reinhardtii* in the form of mature protein of 651 amino acids SEQ ID NO : 5) delimited by the amino acids located at positions 58 and 708 of the peptide sequence shown in Fig. 2,

. the sequence SEQ ID NO : 6 delimited by the nucleotides located at positions 186 to 1499 of SEQ ID NO : 1, coding for a fragment of 438 amino acids (SEQ ID NO : 7) delimited by the amino acids located at positions 58 and 495 of the peptide sequence of the GBSSI of *Chlamydomonas reinhardtii* shown in Fig. 2,

. the sequence SEQ ID NO : 8 delimited by the nucleotides located at positions 186 to 1778 of SEQ ID NO : 1, coding for a fragment of 531 amino

acids (SEQ ID NO : 9) delimited by the amino acids located at positions 58 and 588 of the peptide sequence of the GBSSI of *Chlamydomonas reinhardtii* shown in Fig. 2,

– or a nucleotide sequence derived by degeneration of the genetic code of the aforementioned nucleotide sequences, and coding for the aforementioned GBSSI of *Chlamydomonas reinhardtii*, or for an aforementioned peptide fragment of the latter,

– or a nucleotide sequence derived from an aforementioned nucleotide sequence or fragment, especially by the substitution, suppression or addition of one or more nucleotides, and encoding a peptide sequence derived from the aforementioned GBSSI of *Chlamydomonas reinhardtii*, or derived from an aforementioned peptide fragment of the latter, and having the property of attaching to the starch granules, the said derived nucleotide sequence preferably having a homology of at least about 50%, and preferably of at least about 70%, with the aforementioned nucleotide sequence or fragment,

– or a nucleotide sequence capable of hybridizing with one of the aforementioned nucleotide sequences or fragments, especially in the strict conditions of hybridization defined above.

The invention also relates to the polypeptides selected from:

– the peptide sequence SEQ ID NO : 3 delimited by the amino acids located at positions 1 to 708 in Fig. 2, corresponding to the GBSSI of *Chlamydomonas reinhardtii* in the form of pre-protein of 708 amino acids,

– any fragment as defined above of the peptide sequence SEQ ID NO : 3 shown in Fig. 2, and more particularly any sequence whose amino acid of the amino terminal end corresponds to that located in one of the positions 1 to 58 of SEQ ID NO : 3, and whose amino acid of the carboxy terminal end corresponds to that located in one of the positions 495 to 708 of SEQ ID NO : 3, especially:

. the sequence SEQ ID NO : 5 delimited by the amino acids located at positions 58 to 708 of SEQ ID NO : 3, corresponding to the GBSSI of *Chlamydomonas reinhardtii* in the form of mature protein of 651 amino acids,

. the sequence SEQ ID NO : 7 delimited by the amino acids located at positions 58 to 495 of SEQ ID NO : 3, corresponding to a fragment of 438 amino acids of the peptide sequence of the GBSSI of *Chlamydomonas reinhardtii* shown in Fig. 2,

. the sequence SEQ ID NO : 9 delimited by the amino acids located at positions 58 to 588 of SEQ ID NO : 3, corresponding to a fragment of 531 amino

acids of the peptide sequence of the GBSSI of *Chlamydomonas reinhardtii* shown in Fig. 2,

– or a peptide sequence derived from an aforementioned sequence or peptide fragment, especially by substitution, suppression or addition of one or more amino acids, and having the property of attaching to the starch granules, the said derived peptide sequence preferably having a homology of at least about 60%, advantageously at least about 80%, with the aforementioned peptide sequence or fragment,

the property possessed by the GBSSI of *Chlamydomonas reinhardtii*, or a fragment or a protein derived from the latter as defined above, of being able to attach to the starch granules, being measurable by the method described above.

The invention also relates to polyclonal or monoclonal antibodies, directed against the aforementioned polypeptides.

The invention will be further illustrated by means of the following detailed description of cloning of the gene coding for the GBSSI of *Chlamydomonas reinhardtii*, and obtaining transformed starch granules containing a fusion polypeptide with the said GBSSI, as well as by means of Fig. 1 showing the nucleotide sequence and the protein sequence deduced from the cDNA insert of the CD142 clone coding for the GBSSI of *Chlamydomonas reinhardtii* (the underlined sequence corresponds to one of the three regions that are highly conserved across all the starch and glycogen synthases and is probably involved in fixation of the ADP-glucose substrate).

I) Cloning of the cDNA (complementary DNA) and gDNA (genomic DNA) sequences corresponding to the structural gene of the GBSSI of *Chlamydomonas reinhardtii*.

A) Cloning of the cDNA

The strategy developed for cloning the cDNA corresponding to the structural gene of the GBSSI of *Chlamydomonas reinhardtii* makes use of screening of an expression library using a polyclonal antiserum. The antiserum is able to recognize a polypeptide sequence encoded by a cDNA expressed from a suitable cloning vector.

a) Production of the antiserum

In order to produce an antiserum capable of specifically recognizing the GBSSI of *C. reinhardtii*, the starch obtained from the wild strain (137C) was injected on three

occasions into an albino New Zealand hybrid rabbit. In a similar experiment, the residual starch from a double mutant strain at loci *STA2* and *STA3* (IJ2) was injected in the rabbit in the same conditions.

Detailed protocols:

– Genotypes of the strains of *C. reinhardtii*:

137C : *mt–nit1 nit2*

IJ2 : *mt–nit1 nit2 sta2-29::ARG7 sta3-1*

The 137C strain is the reference strain for all the studies of starch metabolism carried out in *C. reinhardtii*. The IJ2 strain was fully described by Maddelein et al. in 1994. In this double mutant strain at the *STA2* and *STA3* loci, the GBSSI and SSII activities are absent simultaneously. The mutation at the *STA2* locus was generated by gene interruption by means of the pARG7 plasmid (Maddelein et al., 1994) and leads to complete disappearance of the GBSSI from the starch granule, whereas the mutant allele of the *STA3* gene was generated by mutagenesis by X-rays (Fontaine et al., 1993).

– Conditions for culture, extraction and purification of the starch: the cells were cultured for 3 days in the TAP medium with continuous illumination (3000 lux) from an inoculum of $5x10^4$ cells/ml. The main culture is stopped when the cell concentration reaches about $2x10^6$ cells/ml.

Composition of the TAP medium (values for one litre of medium):

| | | | |
|---|---|---|---|
| $NH_4Cl$ | 0,40 g | $ZnSO_4.7H_2O$ | 22 mg |
| Tris | 2,40 g | $H_3BO_3$ | 11,4 mg |
| $KH_2PO_4$ | 0,32 g | $MnCl_2.4H_2O$ | 5,1 mg |
| $K_2HPO_4$ | 1,47 g | $FeSO_4.7H_2O$ | 4,2 mg |
| $CaCl_2.2H_2O$ | 0,05 g | $MoO_3$ | 1,8 mg |
| $MgSO_4.7H_2O$ | 0,30 g | $CoCl_2.6H_2O$ | 1,6 mg |
| EDTA | 50 mg | $CuO_4.5H_2O$ | 1,6 mg |

The pH of the medium is adjusted to 7 with glacial acetic acid

The TAP-N medium has the same base composition, but this medium differs from the first by the absence of nitrogen supplied in the form of ammonium chloride, which is replaced with sodium chloride at the same concentration; it is in these culture conditions that the cells accumulate a quantity of starch representing up to twenty times

that of cells cultivated in TAP medium. In this case, culture is conducted for 5 days in continuous light starting from a culture inoculated at $5 \times 10^5$ cells/ml.

The cells are then concentrated by centrifugation at $2\text{-}4\times10^8$ cells/ml (Tris/acetate buffer pH 7.5 50 mM; EDTA 10 mM; DTT 2.5 mM) then subjected to the action of a French press at 10000 psi. The extract obtained at press discharge is centrifuged at 5000 g for 15 min at 4°C. The deposit containing the starch is resuspended in one volume of water, to which are added nine volumes of Percoll (Pharmacia, Uppsala, Sweden) before being centrifuged at 10000 g for 30 min at 4°C. The Percoll forms a density gradient during centrifugation. The starch, which has a high density (1.3 to 1.5), settles to the bottom of the tube whereas the lipids and other cell debris of low density form a "cap" at the surface of the Percoll gradient. The starch deposit is then rinsed three times with deionized water then stored at 4°C after removing from it the last supernatant from rinsing.

– Conditions for immunization of the rabbit, taking and preparation of the antiserum: the rabbit used in this experiment is an albino New Zealand hybrid rabbit. Three successive injections were made at intervals of three weeks with 20 mg of purified starch suspended in 500 µl of water. 500 µl of standard Freud adjuvant was added to this suspension. Blood samples were taken from the rabbit 3 weeks after the last injection. The serum is prepared by the single centrifugation of the blood after 24 hours of coagulation at 4°C. The antisera generated by the injections of the starches of the 137C and IJ2 strains are identified in the following by the designations "antiserum SA137C" and "antiserum SAIJ2" respectively.

b) Preparation and screening of the cDNA library

The cDNA library was produced from mRNA's purified from the wild strain of *C. reinhardtii*. The λ ZAP expression vector was used.

Detailed protocols:

– Preparation of the complete RNA's of *C. reinhardtii*: this method is an adaptation of the method used for extracting RNA's from the leaves of *Arabidopsis thaliana*. The cells of a culture of $1\text{-}2 \times 10^6$ cells/ml are harvested by centrifugation at 3500 g for 15 min at 4°C. The cells are then divided into aliquots with a volume of about 200 µl. At this stage, the cells are frozen in liquid nitrogen and can be stored at

-80°C for several months. 400 µl of "Z6" buffer of the following composition is added to the 200 µl of frozen cells:

| | | |
|---|---|---|
| Buffer Z6: | MES/NaOH pH 7.0 | 20 mM |
| | EDTA | 20 mM |
| | Guanidine-HCl | 6 M |
| | β-Mercaptoethanol | 100 µM. |

The mixture is stirred very vigorously for several minutes, then 400 µl of phenol/chloroform/isoamyl alcohol mixture (25v/24v/1v) is added and the mixture is stirred vigorously again for several minutes. The whole is centrifuged at 13000 g for 10 min at 4°C. After recovering and then estimating the volume of the supernatant, 1/20 volume of acetic acid at 1 M as well as 0.7 volume of 100% ethanol are added. The nucleic acids are given time to precipitate at -20°C for at least 30 min. After centrifugation at 13000 g for 15 min at 4°C, the pellet is resuspended in 400 µl of 3 M sodium acetate pH 5.6 then centrifuged for 10 min at 13000 g at 4°C. The pellet is then rinsed twice with 70% ethanol, dried and finally dissolved in 50 µl of water treated with DEPC. The quantity of nucleic acids is determined in a spectrophotometer at 260 nm ($OD_{260}$=1 is equivalent to about 40 µg/ml of nucleic acids).

– Construction of a cDNA library in the λ ZAP vector: the RNA's having a polyA tail (the mRNA's in particular) are isolated from the total RNA preparation using the kit "polyATtract mRNA isolation systems" marketed by Promega (Madison, WI, USA). Synthesis of the cDNA's, ligation in the λ ZAP vector and packaging in the capsids are effected using the kit "cDNA synthesis kit, ZAP-cDNA synthesis kit and ZAP-cDNA gigapack II gold cloning kit" marketed by Stratagene (La Jolla, CA, USA). The procedure followed corresponds to the instruction manual supplied with the kit.

– Immunological screening of a cDNA library in an expression vector: screening of the cDNA expression library of λ ZAP from *C. reinhardtii* was carried out using the antiserum previously obtained (see above). About 100000 lysis plates are spread by the Top-agar technique on several Petri dishes containing bacterial growth medium and the adapted antibiotic. After incubation for 3 hours at 37°C, nitrocellulose filters (Protan BA 85, Schleicher & Schuell, Dassel, Germany), previously immersed in a solution of IPTG 10 mM and dried, are applied to the surface of the Top-agar. The dishes are incubated again for 3 hours at 37°C before being stored at 4°C for 30 min. The

nitrocellulose filters are then carefully removed from the agar surface. The *E. coli* strain XL1-blue was used during screening of the λZAP library. The protocol for filter development is then the same as that used in the Western Blot study (see the section dealing with Western Blot).

The positive lysis regions are subjected to two successive series of screening with the same antiserum in order to confirm their positive character, and also to purify them. When a lysis region is found to be positive at the end of three screenings, the sequence of the plasmid pBluescript SK+ containing the insert of interest is excised from the λ phage *in vivo*. It is the "ExAssist helper phage" that is used for cotransfection of the SOLR strain with the λ ZAP phage. In this way we obtain a phagemid that is used for infecting the strain XL1-Blue MRF' leading to restoration of the double-stranded plasmid pBluescript SK+ bearing the cDNA of interest.

Screening of this kind, conducted with the SA137C antiserum, led to the production of a single positive clone after three screenings. We designated this clone "CD142". The insert of the CD142 clone has a size of 1696 bp (see the sequence in Fig. 1).

c) Sequence analysis of the insert of the CD142 clone

When the protein sequence libraries are interrogated with the sequence derived from the cDNA clone "CD142", the greatest similarities are obtained with the GBSSI of the higher plants. This first indication of the origin of this cDNA is reinforced by the presence of an extension of 119 amino acids (about 14 kDa) in the carboxy terminal position of the coding sequence, relative to the main GBSSI's of the higher plants. In fact, the molecular weight of the GBSSI of *C. reinhardtii*, determined by SDS-PAGE, is on average 10 to 15 kDa higher than that of the corresponding proteins in plants. The 119 amino acid extension might explain this difference in molecular weight between GBSSI's of different origins. Taken separately, this extension of the coding sequence does not share any similarity with other known types of polypeptide sequences.

The presence of the UAA stop codon in position 717 signals the start of a very long non-coding region of 946 bp. These noncoding regions in 3' terminal position, which frequently occur in the nuclear genes of Chlamydomonas, seem in particular to be intended to stabilize the messenger.

B) Cloning of gDNA

The gDNA relating to the CD142 clone was isolated after screening an indexed gDNA library in cosmids (Zhang et al., 1994). Constructed in a cosmid vector derived from c2RB, this gDNA library is contained in 120 96-well microtitration plates. Each well (apart from two, to facilitate orientation of the plate) contains a bacterial clone transporting a single cosmid. The whole library thus represents 11280 clones for which the average size of the inserts is approx. 38 kb. The nuclear genome of *C. reinhardtii* is therefore represented there statistically about four times.

Screening of this library with a probe corresponding to the CD142 clone led to the isolation of a genomic DNA clone designated 18B1. The insert present in this single cosmid was analysed in more detail. After restriction by NotI then hybridization with the CD142 probe, only a band of about 9 kb remains positive, indicating that all the information corresponding to the CD142 clone is present in this fragment. The genomic sequence corresponding to the CD142 clone is presented below.

Detailed protocols:

– Preparation of nylon filters for screening: the nylon filters (Hybond N, Amersham Buchler, Braunschweig, Germany) are carefully placed on a Petri dish containing a rich bacterial growth medium supplemented with the appropriate antibiotic (in the present case, ampicillin is used). Each E. coli clone contained in the library is then replicated directly on the nylon filter using a replicating apparatus and the dishes thus prepared are incubated overnight at 37°C. The filters are then removed from the agar surface and subjected to the following treatment:

(1) 2 min with a denaturing solution (NaOH 0.5 M; NaCl 1.5 M)

(2) 2 min with a neutralizing solution (Tris/HCl pH 7.0 0.5 M; NaCl 1.5 M)

(3) 2 min with a rinsing solution (buffer 2 × SSC)

Finally the filters are incubated in a drying cabinet for 2 hours at 80°C.

– Prehybridization and hybridization of the filters: prehybridization is carried out in the hybridization buffer at 42°C for at least 4 hours. Hybridization is effected at 42°C for a whole night in the presence of the $^{32}$P-labelled nucleotide probe. The membrane is washed at 60°C in the washing solution, adjusted to the stringency that we wish to apply. The time and frequency of replacement of the washing baths vary depending on the stringency and the radioactivity levels detected on the membrane. In general, the baths are renewed every 10 min and washing begins with a washing buffer of low

stringency and ends with a buffer of greater stringency. A Kodak X-OMAT AR film is finally exposed to the filters at -80°C in order to detect the positive clones.

Composition of the solutions and buffers:

Buffer SSC × 20: Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of water. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Make up to 1 litre with water.

Hybridization buffer:

| | |
|---|---|
| Formamide | 50% |
| Denhardt's | × 5 |
| SDS | 0.5% |
| Na phosphate buffer pH 7.0 | 50 mM |
| DNA of salmon sperm | 100 µg/ml |
| Bovine serum albumin | 0.5% |

Denhardt's reagent × 100 (quantity for 500 ml in water):

| | |
|---|---|
| Ficoll 400 | 10 g |
| PolyVinylPyrrolidone 40 (PVP40) | 10 g |
| BSA | 10 g |

Phosphate buffer pH 7.0 at 1 M (quantity for 100 ml of buffer):

| | |
|---|---|
| $Na_2HPO_4$ 1 M | 57.7 ml |
| $NaH_2PO_4$ 1 M | 42.3 ml |

Washing buffers:

| | |
|---|---|
| low stringency: | SSC × 2; SDS 0.2% |
| medium stringency: | SSC × 1; SDS 0.5% |
| high stringency: | SSC × 0.5; SDS 0.5% |
| | SSC × 0.1; SDS 0.5% |

– Preparation and labelling of a nucleotide probe with $^{32}P$: the fragment serving as nucleotide probe is generally inserted in the multiple cloning site of a bacterial plasmid. It is therefore first necessary to digest it with the appropriate restriction endonucleases then separate the fragment of interest from the rest of the plasmid by electrophoresis on 1% agarose gel buffered with TAE buffer × 1. The band

corresponding to the fragment of interest is then cut out of the gel and DNA extraction is effected with the kit "The GENECLEAN II Kit" marketed by BIO 101 Inc. (La Jolla, CA, USA). The piece of agarose is firstly dissolved in a 6 M sodium iodide solution. On completion of solution, the DNA molecules are then captured with a silica matrix designated "Glassmilk". The DNA molecules, in the presence of the NaI chaotropic agent, are adsorbed specifically on the silica beads. After eliminating the salts and the dissolved agarose, the DNA molecules are eluted from the silica beads in the presence of sterile water.

Labelling of a nucleotide probe with $^{32}P$ is accomplished using the "Random primed DNA labelling kit" from Boehringer (Mannheim, Germany). The principle is random priming of the elongation reaction by Klenow DNA polymerase using a mixture of hexanucleotides representing all the possible combinations of sequences. The radioactive element is incorporated starting from [$\alpha$-$^{32}P$]-dCTP (3000 Ci/mmol) of which 50 $\mu$Ci is used for each labelling reaction. The radiolabelled probe is finally added to the hybridization solution after denaturing at 95°C for 4 min.

C) The *STA2* locus in *C. reinhardtii* represents the structural gene of the GBSSI

The following analysis demonstrates formally that the *STA2* gene of *C. reinhardtii* corresponds to the structural gene of the GBSSI and that the CD142 clone represents a cDNA that comes from this locus. In fact, restriction analyses of the genomic DNA digested by the BamHI endonuclease reveal a profound change of the restriction profile in the mutant BAFR1 strain at the *STA2* locus generated by gene interruption (Delrue et al., 1992). The same change is also observed in the double mutant IJ2 strain at the *STA2* and *STA3* loci which Maddelein et al. (1994) generated by crossing the BAFR1 strain with a *sta3-1* mutant strain.

Moreover, this change of the restriction profile in the meiotic progeny of the IJ2 strain fused with the "CS9" strain of *C. smithii* could be followed in the following crossing:

| CS9 | × | IJ2 | |
|---|---|---|---|
| (*C. smithii*) | | (*C. reinhardtii*) | |
| +/+ | | *sta2-29::ARG7/sta3-1* | |
| | ↓ | | |
| | +/+ | 25% | |
| | *sta2-29::ARG7/sta3-1* | 25% | progeny |
| | *sta2-29::ARG7/+* | 25% | meiotic |
| | *+/sta3-1* | 25% | |

352 segregants resulting from this crossing were purified, amplified and their starch accumulation phenotype was analysed. 54 meiotic recombinants underwent restriction analysis: 21 of genotype *sta2-29::ARG7/+*, 19 of genotype *+/sta3*-1 and 14 wild. With regard to the 21 segregants of genotype *sta2-29::ARG7/+* their restriction profile, obtained by digestion with BamHI and hybridized with the CD142 probe, still has the same change as the parental strain IJ2. We deduce from this that the *STA2* gene and the CD142 probe are very strongly linked genetically. There is no longer any doubt as to the nature of the CD142 clone, which represents the structural gene of the GBSSI (the *STA2* locus).

Detailed protocols:

Carrying out the crossings: before carrying out the fusion of cells with opposite sexual polarities, it is necessary to put them in a state that is favourable to their fusion. Thus, the cells must first be differentiated into gametes before they are put in direct contact. Gametogenesis is induced in *Chlamydomonas* by subjecting the cells to a nitrogen deficit and in the presence of a strong light source (5000 lux). For this, fresh cells cultivated on rich agar medium (culture of less than 5 days) are suspended in 2 ml of TAP-N medium and left for at least 12 hours in strong light without agitation. The state of the cells is examined with an optical microscope before being brought into contact. After differentiation into gametes, the cells are smaller and in particular much more active than in the case of a non-deficient culture. Equivalent quantities of cells of each sexual polarity are mixed. Fusion is always carried out in strong light. After one hour of contact, cell fusions are already visible in the optical microscope. Analysis of the meiotic segregants will consist of depositing the products of cellular fusion on a rich medium at 4% agar. The dishes thus obtained are incubated in diffuse light for 15 hours

then stored in total darkness for at least a week. This permits maturation of the zygotes and their "encystment" in the 4% agar. After this period of incubation in darkness, the dishes are returned to the light and the following stages are carried out as quickly as possible. In order to eliminate the greatest possible number of unfused vegetative cells, the surface of the agar is scraped very lightly with a razor blade. Observing with a binocular magnifier, a region containing about fifty zygotes is marked off and these are transferred to a fresh dish of rich medium at 1.5% agar. To be sure of complete disappearance of residual vegetative cells, the dish is subjected to chloroform vapours for 45 seconds to 1 minute (in contrast to other cells, zygotes can withstand this moderate time of exposure to chloroform vapours). The presence of light will irreversibly trigger the start of meiosis of the zygotes. During their germination (facilitated by the higher moisture content of the medium containing 1.5% agar) the zygotes will release four haploid daughter cells (a tetrad), which will grow by mitotic divisions and form colonies in the dish. Analysis of the meiosis products can be effected in two ways. The first consists of random investigation of at least 200 segregants resulting from the crossing. After purification of the segregants, the characters of the latter can be studied by replication on different selective media.

Techniques of extraction of genomic DNA: the protocol adopted for extraction of total DNA is that described by Rochaix et al. (1991); here are the details:

(1) Centrifuge 10 ml of cell culture to about 3-5 × $10^6$ cells/ml for 10 min at 3500 g in a 15 ml bottle.

(2) The pellet of cells is then resuspended in 350 µl of the following buffer:

| | |
|---|---|
| Tris/HCl pH 8.0 | 20 mM |
| EDTA | 50 mM |
| NaCl | 100 mM. |

(3) Add 50 µl of proteinase K from a stock solution at 2 mg/ml (if unavailable, it is possible to use pronase at 10 mg/ml).

(4) Add 25 µl of SDS at 20% and incubate for 2 hours at 55°C.

(5) Add 2 µl of diethylpyrocarbonate (DEPC) and incubate for 15 min at 70°C.

(6) Cool the tube briefly in ice and add 50 µl of 5 M solution of potassium acetate.

(7) Mix, by shaking the tube correctly, and leave on ice for at least 30 min (it is possible to stop the extraction at that moment and resume on the next day if the tubes are left in ice in a coldroom).

(8) Transfer to a 1.5ml Eppendorf tube and centrifuge for 15 min in a minicentrifuge (at about 13000 g).

(9) Recover the supernatant, transferring it to a new Eppendorf tube.

(10) Extract the supernatant with one volume of the following mixture:

| | |
|---|---|
| Phenol (saturated with TE : Tris/HCl pH 8.0 10 mM, EDTA 1 mM) | 25 vol |
| Chloroform | 24 vol |
| Isoamyl alcohol | 1 vol |

(11) After extraction, add 1 ml of 100% ethanol at room temperature. A precipitate should be seen to appear in the form of "angel hair" if extraction is successful. From this moment, manipulations must be careful and gentle so that the DNA molecules do not break.

(12) Centrifuge for 5 min in a minicentrifuge (about 13000 g).

(13) Rinse the pellet with 70% ethanol and centrifuge for 3 min in a minicentrifuge.

(14) Repeat operation (13) once or twice for proper elimination of the salts.

(15) Dry the pellet for 5 min at 37°C, then dissolve it in 50 µl of TE containing bovine pancreas RNase at 1 µg/ml.

Molecular hybridizations and Southern Blot analyses: 25 µg of DNA is digested completely with the appropriate restriction endonuclease(s). The restriction products are then separated by electrophoresis in 0.8% agarose gel, TBE × 1. Then the gel is incubated successively for 15 min in the depurination solution and for 30 min in the denaturing solution. The denatured DNA is then transferred onto "Porablot NYplus" nylon membrane (Macherey-Nagel GmbH, Düren, Germany) by capillarity with SSC buffer × 20. After transfer, the membrane is incubated at 80°C in absence of air for 2 hours to fix the DNA fragments to the surface of the nylon membrane. Prehybridization is effected in the hybridization buffer at 42°C for at least 4 hours. Hybridization is accomplished at 42°C for a whole night in the presence of the labelled probe prepared previously. The membrane is washed at 60°C in the washing buffer adjusted to the stringency that is to be applied to the washing. The time and frequency of replacement of the washing baths vary depending on the stringency and the levels of radioactivity present on the membrane. In general, the baths are renewed every 10 min and washing begins with a washing buffer of low stringency and ends with a buffer of higher

stringency. A Kodak X-OMAT AR film is finally exposed to the membrane at -80°C to reveal the hybridization zones.

II) Investigation of binding of the GBSSI to the starch granule.

A) Analysis of the *sta2-1* mutant allele

Among all the mutant alleles generated at the *STA2* locus in *C. reinhardtii*, just one leads to the production of a 58 kDa truncated GBSSI in place of the 76 kDa wild protein. This is the *sta2-1* allele of the 18B strain. Delrue et al. (1992), by micro-sequencing of the GBSSI extracted from a polyacrylamide gel, were able to demonstrate that the amino terminal peptide sequences of the proteins of the wild strain (137C) and of the mutant strain (18B) are identical.

Amino terminal sequences:

- GBSSI of the 137C strain: ALDIVMVAAEVAPGGKTGGLGDV
- GBSSI of the 18B strain: ALDIVMVAAEVAPGGKTGGLGDV

The protein produced by the *sta2-1* mutant allele is therefore truncated in the carboxy terminal position and the $K_m$ for ADP-glucose is increased by a factor of 6. Absence of this carboxy terminal sequence does not, however, alter the properties of fixation of the protein on the granule, as is shown in Fig. 1.

Detailed protocol:

Technique of extraction of the proteins from the starch granule and SDS-PAGE: the proteins are extracted from 0.3 to 1 mg of starch with 60 µl of extraction buffer: β-mercaptoethanol 5% (v/v); SDS 2% (w/v) at 100°C for 5 min. After centrifugation at 13000 g for 10 min, the supernatant is recovered and the operation is repeated once with the pellet. The two supernatants are combined and the sample can be loaded into the gel wells after adding again 10 µl of the following loading buffer: Tris 50 mM, glycine 384 mM, 20% glycerol, SDS 0.1%, bromophenol blue 0.001%. Migration is carried out at room temperature, at 150 V for 1 h 30 (until the bromophenol blue leaves the gel). The proteins are revealed by staining with Coomassie blue or by immunodetection (see below; section relating to Western Blot). During staining with Coomassie blue, the gel is incubated for 30 min in the following solution: 2 g of Coomassie Brilliant Blue R250, 0.5 g of Coomassie Brilliant Blue G250, 425 ml of ethanol, 50 ml of methanol, 100 ml of acetic acid; water sufficient for 1000 ml. The gel is then decolorized using the following solutions:

➢ 15 to 30 min in decolorizer I: 450 ml of ethanol, 50 ml of acetic acid; make up to 1000 ml with water.

➢ one night in decolorizer II: 80 ml of acetic acid, 50 ml of methanol, make up to 1000 ml with water; this decolorizer II removes the nonspecific coloration of the gel.

➢ decolorizer III (240 ml of acetic acid, 200 ml of methanol, make up to 1000 ml with water) permits complete decolorizing of the gel if necessary.

B) Determination of the quantity of proteins bound to the granule

The quantity of proteins bound to the starch granule was determined in different culture conditions and in various gene libraries. For this, the cells were placed in conditions of massive accumulation of starch (nitrogen-deficient medium) or in conditions of mixotrophic growth (presence of nitrogen). The proteins extracted from the granule were then deposited on polyacrylamide gel in denaturing conditions (presence of SDS). After migration, the proteins are revealed by staining with Coomassie blue. The I7 strain, mutant at the *STA1* locus, was used during this experiment. This mutation was described in detail by Van den Koornhuyse et al. (1996) and then by Van de Wal et al. (1998). The *STA1* locus corresponds to the structural gene of the large regulatory subunit of ADP-glucose pyrophosphorylase. The *sta1-1* mutation produced during X-ray mutagenesis leads to insensitivity of the enzyme to 3-phosphoglyceric acid, its allosteric activator. Consequently, the I7 strain accumulates less than 5% of the normal quantity of starch. The estimate of the quantity of GBSSI bound to the granule is approx. 0.1% of the weight of starch in conditions of nitrogen deficiency for the 137C and 18B strains. This value reaches 1% in conditions of mixotrophy. In the case of the I7 strain, regardless of the culture conditions, the GBSSI represents more than 1% of the weight of the starch granule. The techniques employed in this analysis are the same as those described in the preceding paragraph.

C) Analysis of immune response in Western Blot

To test the antigenicity of the SA137C and SAIJ2 antisera obtained previously in the rabbit, the proteins extracted from 100 µg of fresh starch obtained from different strains cultivated in variable culture conditions were subjected to analysis by the immunotransfer technique (Western Blotting). The immune response produced with respect to GBSSI during injection of the starch from the wild strain (137C) proves very specific and strong (the proteins having been extracted from just 100 µg of fresh starch) even in the case of the truncated protein in the *sta2-1* mutant. The quantity of proteins

bound to the starch granule seems larger in the I7 mutant during nitrogen-deficient culture, as shown by the presence of a mass band higher than GBSSI revealed by the SA137C antiserum. This is confirmed by Western Blot analysis effected with the SAIJ2 antiserum, where the strongest immune response is detected with the proteins extracted from the starch of the I7 strain cultivated with nitrogen deficiency.

For control purposes, we carried out the same type of experiment using the PA55 antiserum obtained by Abel et al. (1995). This antiserum produced in the rabbit is directed against a peptide whose consensus sequence corresponds to the strongly conserved carboxy terminal region in all the starch synthases of higher plants, whether they are soluble or bound to the starch granule. This antiserum recognizes the GBSSI of *C. reinhardtii* specifically when the latter is present in the granule. Moreover, the PA55 antiserum also recognizes the truncated protein produced by the 18B mutant (*sta2-1*). It therefore appears that the highly conserved sequence in carboxy terminal position is still present in the truncated protein.

Detailed protocols:

Technique of protein extraction from the starch granule and SDS-PAGE: these techniques are the same as those described in the preceding section apart from staining with Coomassie blue, which is omitted in this case.

Technique of transfer and detection with antisera: when migration on SDS-PAGE has ended, the gel is incubated for 30 min in the "Western" buffer × 1 containing 20% of methanol. The proteins are then electrotransferred onto a nitrocellulose membrane (Protan BA 85 Schleicher & Schuell, Dassel, Germany) using electrotransfer apparatus (Multiphor II, LKB-Pharmacia, Bromma, Sweden) at 4°C in the following conditions: 45 min at 250 mA with the buffer used previously. After this stage of transfer of the proteins onto the nitrocellulose membrane, the latter is incubated for 1 hour at room temperature in TBST buffer containing 3% of BSA. The membrane is then rinsed three times in TBST buffer before being incubated overnight at 4°C in the rabbit primary antiserum diluted in TBS buffer. The membrane is again rinsed three times with TBST buffer and is then incubated for 1 hour at room temperature with the biotinylated secondary antibody diluted at 1/500 in the TBS directed against the rabbit antiserum. After three further rinses in TBST buffer, the membrane is incubated for 30 min at room temperature with the streptavidin-alkaline phosphatase complex at 1/3000 dilution in the TBS buffer. Finally, after 3 rinses in TBST buffer, the membranes are developed by

incubation in a diethanolamine buffer containing the substrates of alkaline phosphatase: NBT and BCIP (the incubation time varies depending on the intensity of reaction). The detection kit used is the one offered by Amersham Buchler (Braunschweig, Germany): "Blotting detection kit for rabbit antibodies"

Compositions of solutions and buffers:

| | | |
|---|---|---|
| "Western" buffer × 10: | Glycine | 390 mM |
| | Tris | 480 mM |
| | SDS | 0.375% |
| TBS buffer (Tris Buffer Saline) | Tris/HCl pH 7.5 | 20 mM |
| | NaCl | 500 mM |

TBST buffer (Tris Buffer Saline Tween): TBS + 0.1% (v/v) Tween 20

NBT: Nitro-Blue Tetrazolium in solution in dimethylformamide 70%

BCIP: 5-Bromo-4-Chloro-3-Indolyl Phosphate in solution in dimethylformamide.

III) Targeting of fusion proteins in the starch granule

The specific carboxy terminal extension of the GBSSI of *C. reinhardtii* is not required for targeting the protein to the starch granule *in vivo* as we were able to demonstrate in the previous experiments. The extension of about 16 kDa can be replaced by a peptide sequence of interest, thus permitting its targeting to the very heart of the starch granule.

The various types of vectors that can be constructed for applying this method in higher plants consist of:

– a bacterial selector gene and a bacterial replication origin in order to be able to amplify the plasmid in a suitable bacterial strain

– a selector gene that will permit easy selection of transformant plants

– translational fusion between the coding sequence of the GBSSI and a polypeptide sequence of interest. Two main types of translational fusions may be considered: in the first case, it is the 58 kDa truncated sequence from GBSSI that is fused with the sequence of interest; in the second case, the complete sequence of the GBSSI is employed.

– fusion can be put under the control of a strong constitutive plant promoter, or of an inducible plant promoter, immediately followed by a suitable transit peptide promoting translocation of the fusion protein to the chloroplast.

IV) Protocol for determination of the activity of granule-bound starch synthase:

Add 20 µg of starch to 100 µl of the following reaction mixture:

| | |
|---|---|
| Glycylglycine/NaOH pH 9.0 | 50 mM |
| $(NH_4)_2SO_4$ | 100 mM |
| β-mercaptoethanol | 5 mM |
| $MgCl_2$ | 5 mM |
| Bovine serum albumin | 0.5 mg/ml |
| ADP-glucose | 0.2 mM |
| [U $^4$C]ADP-glucose (235 mCi/mmol) | 2.66 µg |
| Trisodium citrate | 0 or 0.5 M (specified depending on circumstances) |

The reaction is carried out at 30°C for 15 min and is then stopped by adding 3 ml of 70% ethanol. The precipitate obtained is filtered under vacuum on a "Whatman Glass Fibre" filter (Whatman, Maidstone, UK), and rinsed with 4 × 5 ml of 70% ethanol. A Beckman counter is used for radioactivity counting after the filters have been placed in counting phials containing 3.5 ml of scintillation liquid.

V) The methods of starch extraction and purification are as follows:

– in the case of a single-celled green algae such as *Chlamydomonas reinhardtii* (see the method described above)

– in the case of seeds, tubers or any other organ of higher plants:

the organ or the type taken from the plant is properly homogenized (after grinding). The pulverized material thus obtained is rinsed with water through a filter cloth (such as Miracloth Calbiochem, La Jolla, CA, USA). The filtrate is then left to stand for two hours for the starch granules to settle. The sediment is rinsed firstly with several volumes of water then a second time with several volumes of NaCl solution at 0.1 M. The sediment is filtered once again then rinsed twice with ethanol before being dried.

## BIBLIOGRAPHY

Abel G., Springer F., Willmitzer L. and Kossmann J., (1996), *The Plant Journal*, 10 (6): 981-991

An *et al.* (1986), *Plant Physiol.*, **81**, 301-305

Baba T., Nishihara M., Mizuno K., Kawasaki T., Shimada H., Kobayashi E., Ohnishi S., Tanaka K. and Arai Y., (1993), *Plant Physiology*, 103: 565-573

Bevan M. (1984), *Nucleic Acids. Res.*, **12**, 8711-8721

Brodelius *et al.* (1979), *FEBS Letters*, **103**, 93-97

Brodelius (1988), In: Moo-Young M. (Ed), *Bioreactor Immobilized Enzymes and Cells: Fundamentals and Applications*, Elsevier, Londres

Buléon A., Colonna P., Planchot V., Ball S. (1998), *International Journal of Biological Macromolecules*, 23: 85-112

De La Penna *et al.* (1987), *Nature*, **325**, 274-276

Delrue B., Fontaine T., Routier F., Decq A., Wieruszeski J-M, van den Koornhuyse N., Maddelein M-L., Fournet B. and Ball S. (1992), *Journal of Bacteriology*, 174 (11): 3612-3620

Deno *et al* (1987), *J. Plant. Physiol.*, **131**, 315-322

Denyer K., Clarke B., Hylton C., Tatge H. and Smith A., (1996), *Plant, Cell and Environment*, 18: 1019-1026

Denyer K., Hylton C. and Smith A., (1995), *Planta*, 196: 256-265

Dry I., Smith A., Edwards A., Battacharyya M., Dunn P. and Martin C. (1992), *The Plant Journal*, 2 (2): 193-202

Edwards A., Marshall J., Sidebottom C., Visser R., Smith A. and Martin C. (1995), *The Plant Journal*, 8 (2): 283-294

Fontaine T., D'Hulst C., Maddelein M-L, Routier F., Marianne Pépin T., Decq A., Wieruszeski J-M., Delrue B., van den Koornhuyse N., Bossu J-P., Fournet B. and Ball S., (1993),. *The Journal of Biological Chemistry*, 268 (22): 16223-16230

Gao M., Wanat J., Stinard P.S., James M.G., Myers A.M. (1998) *Plant Cell*, 10(3): 399-412

Hovenkamp-Hermelink J., Jacobsen E., Ponstein A., Visser R., Vos-Scheperkeuter G., Bijmolt E., de Vries J., Witholt B. and Feenstra W. (1987), *Theorical and Applied Genetics*, 75: 217-221

Jouanin *et al.* (1987), *Plant Sci.*, **53**, 53-63

MacDonald F. and Preiss J., (1985), *Plant Physiology*, 78: 849-852

Maddelein M-L., Libessart N., Bellanger F., Delrue B., D'Hulst C., van den Koornhuyse N., Fontaine T., Wieruszeski J-M., Decq A. and Ball S., (1994), *The Journal of Biological Chemistry*, 269 (40): 25150-25157

Marshall J., Sidebottom C., Debet M., Martin C., Smith A. and Edwards A., (1996), *The Plant Cell*, 8: 1121-1135

Mu C., Harn C., Ko Y-T., Singletary G., Keeling P. and Wasserman B., (1994), *The Plant Journal*, 6 (2): 151-159

Müller-Röber B., Sonnewald U. and Willmitzer L. (1992), *The EMBO Journal*, 11(4): 1229-1238

Rochaix J., Mayfield S., Goldschlmidt-Clermont M. and Erickson J., (1991), *Plant Molecular Biology : a Practical Approach*, pp 253-275, ed. Shaw C., IRL Press, Oxford

Sanford J.C. (1988), *Trends in Biotechnology*, **6**, 299-302

Shannon J. and Garwood D. (1984), In *Starch: Chemistry and Technology*, 2nd ed., Whistler R., Bemiller J., Paschall E., eds., Academic Press, San Diego, California: 26-86

Smith A., (1990), *Planta*, 182: 599-604

Tsai C-Y., (1974), *Biochemical Genetics*, 11 (2): 83-95

Van den Koornhuyse N., Libessart N., Delrue B., Zabawinski C., Decq A., Iglesias A., Carton A., Preiss J. and Ball S., (1996), *The Journal of Biological Chemistry*, 271(27): 16281-16287

Van de Wal et al (1998), *The Journal of Biological Chemistry*, 273(35): 22232-22240

Zhang H., Herman P. and Weeks D., (1994), *Plant Molecular Biology*, 24: 663-672